Available online at www.sciencedirect.com

ScienceDirect

Procedia Chemistry 1 (2009) 1291–1294

Procedia Chemistry

www.elsevier.com/locate/procedia

Proceedings of the Eurosensors XXIII conference

Detection of pathogenic Bacteria by Electrochemical Impedance Spectroscopy: Influence of the immobilization strategies on the sensor performance

M. Barreiros dos Santos[a,b,c], C. Sporer[a,c,*], N. Sanvicens[c,d], N. Pascual[d,c], A. Errachid[a,c], E. Martinez[a,c], M.-P. Marco[d,c], V. Teixeira[e], J. Samiter[a,c,f]

[a] *Laboratory of Nanobioengineering, Institute for Bioengineering of Catalonia (IBEC), 08028 Barcelona, Spain*
[b] *International Iberian Nanotechnology Laboratory -INL, 4704 Braga, Portugal,*
[c] *Centro de Investigación Biomédica en Red en Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), 50018 Zaragoza, Spain*
[d] *Applied Molecular Receptor Group (AMRg), Institute for Advanced Chemistry of Catalonia (IQAC), Spanish Council for Scientific Research (CSIC), 08034 Barcelona, Spain*
[e] *Department of Physics, University of Minho, 4800 Guimarães, Portugal*
[f] *Departament d´Electrónica, Universitat de Barcelona, 08028 Barcelona, Spain*

Abstract

Electrochemical impedance spectroscopy (EIS) is applied to detect pathogenic *E. coli* O157:H7 bacteria via a label free immunoassay-based detection method. Polyclonal anti-*E.coli* antibodies (PAb) are immobilized onto gold electrodes following two different strategies, via chemical bond formation between antibody amino groups and a carboxylic acid containing self-assembled molecular monolayer (SAM) and alternatively by linking a biotinylated anti-*E. coli* to Neutravidin on a mixed-SAM. Impedance spectra for sensors of both designs for increasing concentrations of *E. coli* are recorded in phosphate buffered saline (PBS). The Nyquist plots can be modeled with a Randle equivalent circuit, identifying the charge transfer resistance R_{CT} as the relevant concentration dependent parameter. Sensors fabricated from both designs are able to detect very low concentration of *E. coli* with limits of detection as low as 10-100 cfu/ml. The influence of the different immobilization protocols on the sensor performance is evaluated in terms of sensitivity, dynamic range and resistance against nonspecific absorption.

Keywords: Biosensors, Impedance spectroscopy, Bacteria detection, E-coli

Introduction

The detection of contamination of food or water resources by pathogenic microorganism is an issue of utmost importance for ensuring food safety, security and public health. *Escherichia coli* O157:H7 is one of the food borne

* Corresponding author. Tel.: +34 93 403 71 78 ; fax +34 93 403 97 02 .
E-mail address: csporer@pcb.ub.es, c.sporer@ub.edu.

doi:10.1016/j.proche.2009.07.322

pathogenic bacteria that are of most concern today since it can cause severe, sometimes life-threatening illness. The rapid, sensitive, and specific detection of pathogenic microorganisms is therefore essential to prevent intoxications and to provide effective treatment to a susceptible population. [1]

However, conventional methods for bacteriological testing usually involve time-consuming microbiological culturing, reason why the development of alternative detection and identification technologies has become increasingly important in recent years. Biosensors techniques are particularly attractive for the detection and identification of pathogenic microorganisms due to their potential sensitivity and specificity. [2]

Here we are applying electrochemical impedance spectroscopy for the affinity- based immunodetection of *E. coli* since it is known as very sensitive technique that allows label free detection.[3] We are comparing two different functionalization strategies, covalent attachment of antibodies through amide bond formation and noncovalently via Neutravidin/ Biotin conjugation, to evaluate their influence on the sensor performance in terms of sensitivity, dynamic range and resistance against nonspecific absorption.

1. Materials and Methods

1.1. Electrochemical analysis

Cyclic voltammetry (CV) and Impedance spectroscopy (EIS) experiments were performed on a VMP2 multi-potentiostat (Princeton Applied Research), using a three electrode electrochemical cell with a gold disc working electrode (BASi), a Pt wire counter, and an Ag/AgCl reference electrode, placed into a Faraday cage. Measurements were performed in PBS solution (non-faradaic) or in PBS / 5mM $K_3[Fe(CN)_6]$ solution (faradaic EIS). Data acquisition and analysis were accomplished using EC-Lab software (Bio-Logic SAS).

1.2. Antibody biotinylation

Antibody against *Escherichia coli* (PA1-7213; Affinity Bioreagents) was biotinylated with an aliquot of Biotin-*N*-hydroxysuccinimide ester (FLUKA) (1/10 the weight of the antibody) in borate buffer (0.2 M boric acid-sodium borate; pH 8.7) for 2h and subsequently dialyzed in PBS (0.5 mM; 3 x 5 L) to remove unreacted biotin. The recognition capability of the antibodies has been verified by classical ELISA technique for *E.coli* O157:H7 (heat killed, KPL 50-95-90).

1.3. Electrode functionalization

Prior to use, gold working electrodes were cleaned mechanically using a polishing kit (BASi) and chemically by immersion into Piranha solution for several minutes (3:7 by volume of 30% H_2O_2 and H_2SO_4, *Caution: piranha solution reacts violently with most organic materials and must be handled with extreme care*), washed extensively with water, absolute ethanol and dried under a stream of nitrogen. Electrodes were also cleaned electrochemically by applying a potential scan in 0.1N H_2SO_4 solution.

Self-assembled monolayers of mercaptohexadecanoic acid (Aldrich) were formed by immersion of the cleaned electrodes into a 1mM ethanolic solution for 16h. Antibodies were attached covalently by amide bond formation to the surface, previously activated with pentafluorophenol (PFP) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimine hydrochloride (EDC) [4]. Subsequently, remaining active ester functionalities were blocked by reaction with 2-(2-Aminoethoxy)ethanol (Aldrich) and the functionalized electrodes were stored in PBS solution.

Alternatively, biotinylated antibodies are conjugated noncovalently to a Neutravidin layer, previously deposited onto a mixed monolayer formed by biotinylated ethyleneglycolalkylmercaptane [5] and tetraethyleneglycolundecylmercaptane (ratio 1:10 w/w, 0.1mM in ethanol, 16h) [6].

2. Results and Discussion

The controlled immobilization of specific antibodies onto the electrode surface is a crucial step in EIS sensor fabrication. The technique is very sensitive to changes on the electrode surface and specificity to a certain analyte has to be achieved by using selective antibodies and by reducing unspecific adsorption of compounds present in a biological sample. Often, unspecific adsorption in biosensors is reduced by application of a blocking agent like the small protein BSA prior to measurements. Chemical modification of sensors with protein repellent surfaces is a very attractive alternative and neutral hydrophilic compounds like oligo ethyleneglycols are especially well suited. [7] Here we are investigating the influence of different immobilization methods on the sensor activity and nonspecific adsorption resistance.

In the first functionalization strategy, a carboxylic acid derivative was self-assembled on the electrode surface and activated by reaction with a carbodiimide. Subsequently, the antibody was attached covalently to the surface via amide bonding. Since this reaction can take place between every amine group, a certain percentage of antibodies will lose its recognition capability. Afterwards, unreacted active carboxylic ester functionalities were chemically deactivated by reaction with ethoxyethylamine.

The second functionalization strategy is based on the strong noncovalent interaction between a biotin derivative and neutravidin, which can bind up to four biotin molecules. First, a mixed monolayer of thiols containing biotin and ethyleneglycol groups was self-assembled on the surface and reacted with Neutravidin. Subsequently, the biotinylated antibody was allowed to react with the remaining binding positions of the Neutravidin. This procedure avoids the chemical deactivation step which could damage the proteins. However, biotinylation of the antibody also occurs unspecific on all amino groups and may result in reduced recognition capability. Anyway, such systems have proved to be excellently suited for biosensor development. [5,6]

The quality of self-assembled monolayers formed onto electrode surfaces was investigated by cyclic voltammetry in the presence of the redox probe Ferrocyanide. Figure 1 show how the electric current through the electrode is effectively reduced in the presence of the monolayers. Such Voltammograms have been recorded for all functionalized electrodes on the different functionalization steps.

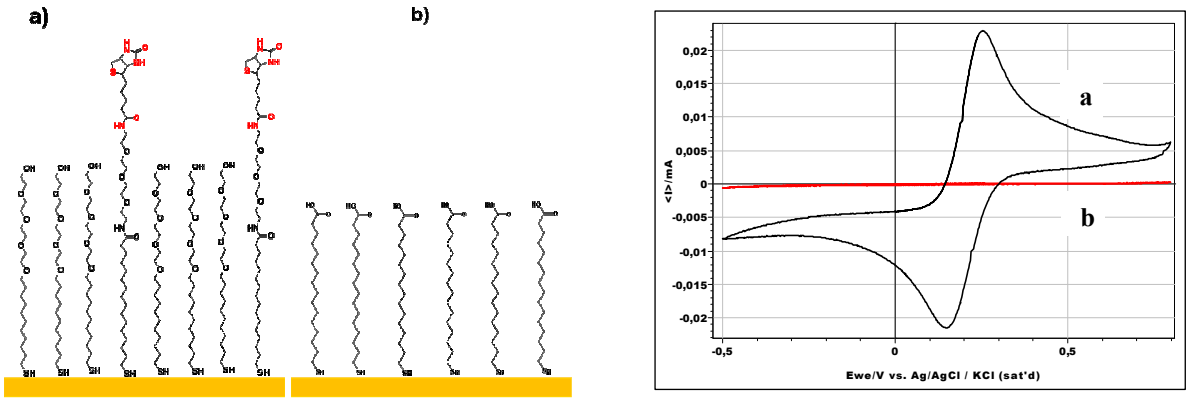

Fig. 1. Left side: Schematic vief of the self-assembled monolayers used for the electrode functionalization a) mixed biotinylated ethyleneglycolalkylmercaptane SAM and b) mercaptohexadecanoic acid.
Right side: Cyclic voltammograms recorded in PBS,containing 5mM $K_3[Fe(CN)_6]$ as redox active probe at scan rates of 100mV/s of a) bare gold electrode and b) gold electrode modified with a SAM of covalently bonded antibody.

Impedance spectroscopy is performed both, in PBS solution alone (non-faradaic) and also in presence of $Fe(CN)_6$ (faradaic EIS) with increasing concentrations of *E. coli*. (Figure 2) and a frequency range between 0,1Hz and 100kHz. The obtained Nyquist plots can be fitted perfectly by a Randle equivalent circuit revealing that R_{CT} is the relevant concentration dependent parameter. Very low concentration of *E. coli* (10-100 cfu/ml) can be detected with sensors fabricated from both designs. (Figure 2)

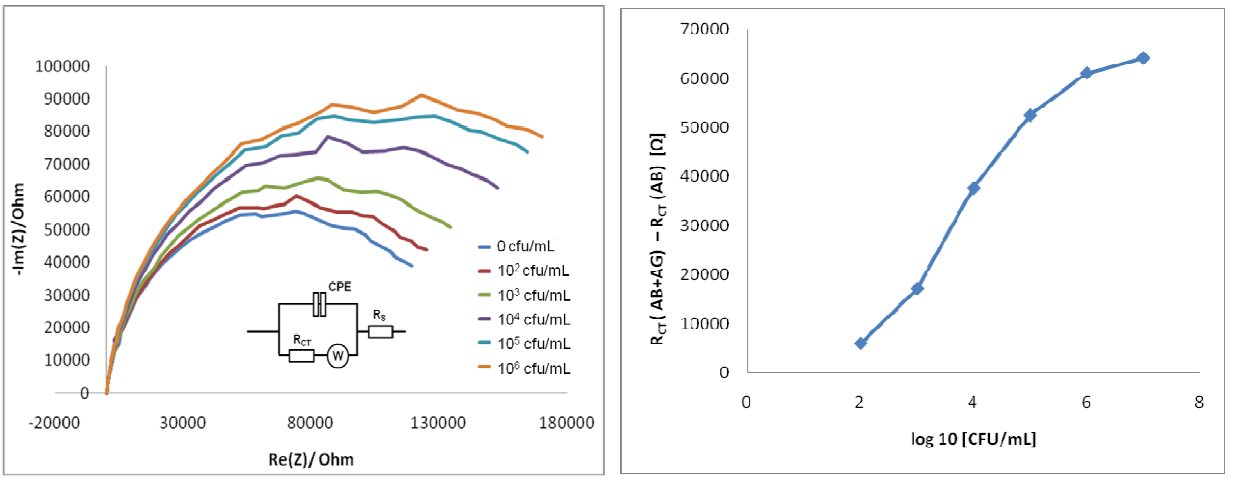

Fig. 2. Left side: Nyquist plots of impedance spectras obtained for increasing concentrations of *E. coli* O157:H7 from 10^2 to10^6 cfu/ml in phosphate buffered saline (PBS) and $Fe(CN)_6$ for a sensor with antibodies immobilized by covalent bonding. Curves are fitted using EC-Lab software (BioLogic Scientific Instruments), best results are obtained with a Randle equivalent circuit (shown as inset) where *CPE* is the constant phase element, R_{CT} the charge transfer resistance, *W* is the Warburg impedance and R_S is the resistance of the solution. Right side: Variation of the charge transfer resistance R_{CT} (solid squares) with the logarithmic concentration of *E. coli* from 10^2 to10^7 cfu/ml for a sensor with antibodies immobilized on the sensor surface by covalent bonding. Values are normalized to R_{CT} in absence of antigen (AG) detection. The data follow a sigmoidal curve (fit R^2= 0.9997).

Currently, the influence of the different immobilization protocols on the overall sensor performance is evaluated in terms of sensitivity and detection limit, dynamic range and sensor saturation, reproducibility and specificity for *E. coli* O157:H7 bacteria.

Acknowledgements

This work is partially funded by the CIBER-BBN under the project MICROPLEX. M.S. thanks the INL for a doctoral grant. C.S. gratefully acknowledges support from the Ministerio de Educación y Ciencia (MEC) via the RyC program.

References

1. *Food safety and foodborne illness*, World Health Organization Fact sheet N°237, 2007, http://www.who.int/mediacentre/factsheets/fs237/en/
2. *Principles of Bacterial Detection: Biosensors, Recognition Receptors and Microsystems* (Ed. M. Zoutrob, S. Elwary, A. Turner) Springer Science+Business Media, LLC, New York, 2008.
3. a) Lisdat F, Schäfer D. *Anal Bioanal Chem* 2008; **391**, 1555–1567. b) Daniels JS, Pourmanda N. *Electroanalysis* 2007; **12**, 1239 – 1257.
4. Hyun J Z Y, Liebmann-Vinson A, Beebe TP, Chilkoti A. *Langmuir* 2001; **17**, 6358-6367
5. a) Vidic J, Pla-Roca M, Grosclaude J, Persuy MA, Monnerie R, Caballero D, Errachid A., Hou Y, Jaffrezic-Renault N, Salesse R, Pajot-Augy E, Samitier J. *Anal Chem* 2007; **79**: 3280-3290. b) Prats-Alfonso E, Garcıa-Martın F, Bayo N, Cruz LJ, Pla-Roca M, Samitier J, Errachid A, Albericio F. Tetrahedron 2006; **62**, 6876–6881. c) Maalouf R, Fournier-Wirth C, Coste J, Chebib H, Sakali Y, Vittori O, Errachid A, Cloarec JP, Martelet C, Jaffrezic-Renault N. *Anal Chem* 2007; **79** (13): 4879-4886.
6. Nelson K, Gamble L, Jung LS, Boeckl MS, Naeemi E, Golledge SL, Sasaki T, Castner DG, Campbell CT, Stayton PS. *Langmuir*; **17**, 2807-2816
7. Herrwerth S, Eck W, Reinhardt S, Grunze M. *J Am Chem Soc* 2003; **125** (31), 9359–9366